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13. ABSTRACT ***(Maximum 200 Words)***

This is a final report correlating loss of SSeCKS (Src Suppressed C Kinase Substrate; pronounced essex) expression with increased metastatic potential in prostate cancer. The expression of SSeCKS, and its human orthologue, Gravin, is severely downregulated in human and rat prostate cancer cell lines compared to immortalized, untransformed prostate epithelial cell lines or prostate tissue sections from normal patients or patients with benign prostatic hypertrophy or low-grade prostate cancers (Gleason <5). SSeCKS/Gravin staining in advanced, undifferentiated human prostate tissue sections (Gleason >6) was severely downregulated. The regulated re-expression of SSeCKS in prostate cancer cell lines suppressed growth and induced differentiation, marked by cell flattening, filopodia formation, cell-cell adhesion and the loss of anchorage-independent growth. In nude mice, SSeCKS re-expression severely decreased the formation of lung metastases. These data strongly suggest that SSeCKS/Gravin encode prostate metastasis-suppressors.

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FINAL REPORT
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INTRODUCTION: The molecular mechanisms leading to prostate cancer remain poorly understood, especially concerning the progression to the metastatic form. SSeCKS (Src-Suppressed C Kinase Substrate; pronounced *essex*), a major protein kinase C substrate with tumor suppressor activity, is the rodent orthologue of human Gravin, a scaffolding protein for protein kinases A and C. SSeCKS was identified originally in differential screens for genes downregulated in *src*- and *ras*-transformed cells that might act as G1→S regulators. Preliminary data indicate that the scaffold activity of SSeCKS/Gravin is inhibited after phosphorylation by mitogen- and integrin-activated kinases.

BODY: We mapped Gravin to 6q24-25.2, a hotspot for deletion in >60% of advanced prostate cancer, and therefore, we investigated the role of SSeCKS/Gravin in prostate oncogenesis. Abundant SSeCKS/Gravin transcription was evident in normal rat and human prostate tissue. SSeCKS/Gravin protein was detected in untransformed rat and human prostate epithelial cell lines EP12 and PZ-HPV-7, respectively, and in human prostatic epithelium, especially basal epithelial cells. In contrast, SSeCKS/Gravin protein and RNA levels were severely reduced in human (PC-3, PPC-1, LNCaP, DU145, TSU) and rat Dunning (AT3.1, MatLyLu) prostate cancer cell lines. Loss of SSeCKS/Gravin expression correlated with increased metastatic growth potential of the cancer cell lines in nude mice or in syngeneic Copenhagen rats. SSeCKS/Gravin deficiency was either due to gene deletion (AT3.1 or LNCaP) or transcriptional downregulation (MatLyLu or PC-3).

The regulated re-expression of SSeCKS in MatLyLu and LNCaP cells was associated with growth suppression and increased differentiation. Specifically, SSeCKS induced cell flattening and the production of filopodia-like projections, and caused a decrease in anchorage-independent growth. Prostate cancer cells with loss in SSeCKS/Gravin expression contained a novel 80kDa isoform of

SSeCKS/Gravin most likely produced by a cancer-specific protease whose activity is blocked in untransformed cells. Additionally, the regulated re-expression of SSeCKS suppressed the generation of the 80kDa isoform. Lastly, in cells showing measurable, although dowregulated levels of SSeCKS, there is an increase in the PKC-mediated phosphorylation, as shown by phospho-specific antibodies we raised to PKC sites on SSeCKS.

In nude mice, SSeCKS re-expression slightly decreased primary-site tumor growth but severely decreased the formation of lung metastases. Primary-site tumors that progressed lost regulated SSeCKS re-expression. SSeCKS/Gravin expression was detected in normal prostate epithelium, in benign human prostatic lesions and well-differentiated carcinomas but not in undifferentiated lesions with Gleason scores >6.

CONCLUSIONS: Overall, our data suggest a role for the loss of SSeCKS/Gravin in the metastatic progression of human prostate cancer. We conclude that SSeCKS/Gravin expression is required for the differentiated phenotype of prostate epithelial cells, including aspects of mitogenic and cytoskeletal control, cell-cell adhesion, and anchorage-dependence. Loss of SSeCKS/Gravin occurs by two mechanisms: gene deletion or transcriptional downregulation. SSeCKS/Gravin is a suitable marker for advanced prostate cancer, either by showing loss of overall expression or a relative increase in the PKC-phosphorylated forms (using our phospho-specific antisera).

KEY RESEARCH ACCOMPLISHMENTS:

-Demonstrated that the "Src-Suppressed C Kinase Substrate", SSeCKS, is a cancer suppressor whose function is attenuated by mitogen- and integrin-activated kinases.

-Showed that expression of SSeCKS, and its human orthologue, Gravin, is downregulated in prostate cancer cell lines (rat and human) and in advanced human prostate cancer tissue sections, compared to normal prostate epithelial cell lines or non-cancerous or low-grade prostate cancer tissues.

-Prepared monoclonal antibodies specific for two phosphorylated SSeCKS domains representing major PKC phosphorylation sites. These reagents will be used in future investigations as possible diagnostic markers for advanced prostate cancer.

-Mapped the human SSeCKS orthologue, Gravin, to 6q24-25.2, a deletion hotspot in advanced prostate, breast, and ovarian cancer.

-Supported the case for SSeCKS/Gravin homology by correlating SSeCKS/Gravin loss with increased metastatic potential in various prostate cancer cell lines, nude mice and rats.

-Showed that the regulated re-expression of SSeCKS suppresses cell growth, induces cell differentiation marked by cell flattening and production of filopodia, and decreases anchorage-independent growth.

-Demonstrated that the level of SSeCKS/Gravin staining is severely decreased in poorly

differentiated human prostate carcinomas as compared with prostatic epithelia from normal, BPH or well-differentiated prostates.

-Associated SSeCKS/Gravin downregulation with the proteolytic production of an 80kDa SSeCKS isoform that may antagonize SSeCKS' anti-metastasis activity. Also identified is a protease inhibitor expressed in untransformed cells whose activity or expression correlates with SSeCKS re-expression.

PRODUCTS FROM THE RESEARCH:

-Hybridomas producing monoclonal antibodies that recognize phospho-Ser^{300} and -Ser^{515} in SSeCKS.

-Immunoaffinity-purified polyclonal sera specific for a "unique" C-terminal domain in Gravin, usable for immunohistochemical analyses of protein expression in human tissues.

-MatLyLu and LNCaP cell lines with tetracycline-regulated expression of SSeCKS.

BIBLIOGRAPHY:

Manuscripts

Xia, W., Unger, P., Miller, L., Nelson, J. and **Gelman, I.H.**, "Suppression of prostate metastasis by re-expression of the *Src*-suppressed C kinase substrate, SSeCKS" (submitted to *J. Clin. Invest.* for publication).

Poster Presentations

"Suppression of prostate metastasis by re-expression of the *Src*-suppressed C kinase substrate, SSeCKS", 91st Annual Meeting, American Association for Cancer Research, San Francisco, CA, April 1-5, 2000.

"Suppression of prostate cancer metastatic potential by the forced re-expression of Src-Suppressed C Kinase Substrate, SSeCKS: Evidence for tumor suppressor activity in humans", AACR 92nd Annual Meeting, New Orleans, March 24-28, 2001.

Seminars

- "Tumor Suppression, Control of Mitogenesis and Cytoskeletal Architecture by SSeCKS, a Major PKC Substrate with Scaffolding Activity", University of Mainz, Frankfurt, Germany, July 2, 1999.
-"A Novel Tumor Suppression Pathway: Control of Cyclin D Expression and Compartmentalization by the Src-Suppressed C Kinase Substrate, SSeCKS", October 22, 1999, Picower Institute, NY; invited seminar.
-"A Novel Tumor Suppression Pathway: Control of Cyclin D Expression and Compartmentalization by the Src-Suppressed C Kinase Substrate, SSeCKS", December 15, 1999, Moffitt Cancer

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The *Src*-Suppressed C Kinase Substrate, SSeCKS, is a Metastasis Suppressor in Prostate Cancer

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ABSTRACT

The molecular mechanisms leading to prostate cancer remain poorly understood, especially concerning the progression to the metastatic form. SSeCKS, a major protein kinase C substrate with tumor suppressor activity, is likely the rodent orthologue of human Gravin, a scaffolding protein for protein kinases A and C. Gravin mapped to 6q24-25.2, a hotspot for deletion in advanced prostate cancer, and therefore, we investigated the role of SSeCKS/Gravin in prostate oncogenesis. SSeCKS/Gravin protein was detected in untransformed rat and human prostate epithelial cell lines EP12 and PZ-HPV-7, respectively, and in human prostatic epithelium, especially basal epithelial cells. In contrast, SSeCKS/Gravin protein and RNA levels were severely reduced in human (PC-3, PPC-1, LNCaP, DU145, TSU) and rat Dunning (AT3.1, MatLyLu) prostate cancer cell lines. The regulated re-expression of SSeCKS in MatLyLu cells induced filopodia-like projections and a decrease in anchorage-independent growth. In nude mice, SSeCKS re-expression slightly decreased primary-site tumor growth but severely decreased the formation of lung metastases. Primary-site tumors that progressed lost regulated SSeCKS re-expression. SSeCKS/Gravin expression was detected in benign human prostatic lesions and well-differentiated carcinomas but not in undifferentiated lesions with Gleason scores >6. Our data suggest a role for the loss of SSeCKS/Gravin in the metastatic progression of human prostate cancer.

INTRODUCTION

Prostatic adenocarcinoma (PA) is the most common non-cutaneous malignancy in men in the U.S., associated with roughly 38,000 deaths in the U.S. in 1994 (1) and 42,000 deaths in 1996 (2). Especially hard hit are African American men whose incidence is highest in the world (2). A critical factor in prostate cancer-related mortality is the metastatic potential of the tumor cells and whether the disease will disseminate to secondary sites such as the femur or pelvis. An even larger number of U.S. men show localized, non-disseminated prostatic cancer with much lower risk of mortality. The high frequency of benign prostatic hyperplasia (BPH) in men is associated with the continuous growth of the prostate with age. However, the link between BPH, non-disseminated PA and malignant PA (MPA) remains unclear.

Much data regarding the molecular biology of prostate cancer development and progression comes from a pedigree of Dunning R-3327 rat prostate cancer lines with varying degrees of tumorigenicity, metastatic potential and androgen-dependency (3,4). For example, the G cancer line is poorly tumorigenic in syngeneic, Copenhagen rats, whereas MatLyLu (MLL) cancer cells induce intra- and extraprostatic primary-site tumors and lungs metastases; the AT-3 -derived lines are highly tumorigenic and metastatic and also androgen-independent.

The molecular pathogenesis of MPA is a multistep process involving the activation of endogenous oncogenes as well as the loss of tumor suppressor/cancer susceptibility genes. No single oncogene has been associated with MPA, yet at least 40% of cases studied contain activating mutations or increased expression of oncogenes such as *ras*, myc or *fos* (3,5). Even more significant is the loss of critical tumor suppressor functions

such as p53, DCC and Rb, or cytoskeletal/adhesion molecules such as E-catenin/α-cadherin (6,7). These losses correspond to allelic deletions in chromosomes 5q, 17p, 18q and 13q, found in a small but significant population of MPAs, and deletions in 10q and 6q, found in >60% of MPAs.

We identified a potential tumor suppressor, SSeCKS, whose expression is downregulated in *src*- and *ras*-transformed fibroblasts (8). Re-expression of SSeCKS suppresses *src*-induced oncogenesis such as growth factor- and anchorage-independence, loss of contact inhibition and metastatic potential, mainly by reorganizing actin-based cytoskeletal architecture (9). SSeCKS is likely the rodent orthologue of human Gravin, a scaffolding protein for PKC and PKA (10). SSeCKS also plays a role in G1→S progression by modulating cyclin D expression and by sequestering G1 phase cyclins in the cytoplasm (11). Here, we map *gravin* to chromosome 6q24-25.2, a hotspot for deletion in advanced prostate cancer (12-21). We show that loss of SSeCKS/Gravin expression is typical in human and rat prostate cancer cell lines, and in undifferentiated human prostate cancers *in vivo*. Moreover, re-expression of SSeCKS in MLL cells suppresses cell rounding, anchorage-independent growth and the generation of secondary lung metastases in nude mice. These data suggest a key role for SSeCKS/Gravin in the growth control of normal prostate epithelial cells.

MATERIALS AND METHODS

Cell culture: MatLyLu (MLL) cells, EP12 (EPYP-1; a gift of K. Pienta, U. of Michigan Comprehensive Cancer Center), LNCaP/tTA (LNGK9; a gift of T. Powell, Memorial-Sloan Kettering Cancer Center; ref. 22), and HeLa (ATCC #CCL2.1) were grown in DME (; GIBCO, Gaithersburg, MD) supplemented with 10% fCS (GIBCO). P69 (P69SV40T), M2182 and M12 (gifts of J. Ware, Medical College of Virginia) were grown in RPMI 1640 plus insulin, transferrin and selenium (Collaborative Biochemicals), dexamethasone (Sigma; St. Louis, MO) and EGF (Collaborative) as described (23).

Production of tetracycline-regulated MLL cell lines: MLL/tTAK cells, expressing a tetracycline (tet)-regulated tTA transactivator (24), were produced by transfecting with $CaPO_4$/DNA precipitates containing 3.5 µg of pTet-tTAK and 0.6 µg of pRSV/*hygro* followed by selection of stable transfectants in 400 µg/ml of hygromycin (Sigma). Individual clones were tested for the ability to induce expression of tet_O/luciferase (pUHD13-3) in the absence of tet (25). Clones #2 and #7 were chosen for secondary transfection with 3.8 µg of pUHD10-3/SSeCKS (26) and 1.6 µg of pBABE/*puro* (27), and stable transfectants were isolated after selection in hygromycin and puromycin (8 µg/ml). All cells were selected in 5 µg/ml tet and then maintained on 0.7 µg/ml.

FISH analysis: FISH analysis was performed by See DNA Biotech, Inc. (Downsview, Ontario). A 6.2kb Gravin cDNA fragment was labeled with Biotin-14-dATP using a BRL BioNick kit according to the manufacturer's specifications. Slides were prepared with human lymphocytes grown in α-minimal essential medium containing 10% fCS, phytohemagglutinin, and bromodeoxyuridine (180 µg/ml; Sigma), then grown for 6 h in medium containing thymidine (2.5 µg/ml). FISH detection was performed as described

previously (28,29).

Colony assay in soft agar: 10^4 cells were plated into soft agar in 6 cm wells as described previously (9) and then grown for 3 weeks at 37°C with bi-weekly media feedings.

Northern and western blotting: Total or poly A-selected RNAs were electrophoresed, blotted and probed with [^{32}P]-rat SSeCKS cDNA as described (8). RIPA lysates containing 40-100 µg of total protein were prepared and immunoblotted using rabbit polyclonal anti-SSeCKS Ig as described (30).

Immunofluorescence (IFA) and immunohistochemistry analyses: Cells seeded onto 22mm^2 coverslips were fixed and stained with immunoaffinity-purified (IAP) rabbit polyclonal anti-SSeCKS (30) or rabbit anti-Gravin sera (31) as described previously (26). Immunochemistry was performed as described (31). Slides were viewed on an Olympus IX-70 fluorescent microscope and digitized using a Sony Catseye camera connected to a PowerMac G3 (Apple Computers). Image analysis was performed using Adobe Photoshop 4.01.

Tumor and metastasis formation in nude mice: Six-week-old female nude mice (Taconic Farms, Germantown, NY) were injected *s.c.* with 10^5 MLL/vector or MLL/SSeCKS clones. The viability of the cells was >90% as determined by trypan blue exclusion. All mice were fed water containing 100 µg/ml tet plus 5% sucrose until the primary tumors were palpable (2-4 mm), at which point, the tet-water was withdrawn. Mice were sacrificed 3 weeks after injection. The primary tumors were measured and weighed, and the lungs were stained for metastases by injecting India ink (30 ml ink plus 4 drops of 1 M ammonium hydroxide, diluted into 200 ml of dH_2O) into the trachea for 10 min at room

temperature followed by several washes in PBS. Surface metastases, which exclude the dye, were then counted.

RESULTS

Mapping of SSeCKS/Gravin. Rodent SSeCKS and human Gravin show 83% identity over the first ~1000 a.a., <20% similarity over the next ~500 a.a., and identity in two 15-a.a. stretches at the C-termini (10). Full-length SSeCKS cDNA recognizes Gravin transcript under conditions of stringent hybridization (31). Using a Gravin cDNA probe, we mapped *gravin* by fluorescence *in situ* hybridization (FISH) to chromosome 6q24-25.2 (Fig. 1). Secondary hybridization signals were not detected which might reflect a second family member. These map coordinates are confirmed by microsatellite markers (Sanger Sequencing Centre, UK). FISH analysis using a full-length SSeCKS cDNA probe identified the same, singular region (data not shown). Deletions in this region are associated with advanced, non-organ confined prostate cancer cases, suggesting a possible role for SSeCKS/Gravin in prostate oncogenesis.

Loss of SSeCKS/Gravin expression in human and rat prostate cancer cell lines. Northern blots containing total or poly A-selected RNA from Dunning rat prostate cancer cell lines or tumors (grown in Copenhagen rats) or from human prostate cancer cell lines were probed under stringent conditions with rat SSeCKS cDNA. Fig. 2A shows a progressive loss of SSeCKS RNA signal in MLL and AT-3.1 cancer cells (5- and >20-fold lower, respectively) compared to the mildly transformed G cell line. AT-3.1 cells, which are androgen-independent, are slightly more metastatic in nude mice than MLL. SSeCKS RNA levels in H-, MatLu-, MLL- or AT-3-induced tumors are reduced >5-fold compared to levels in the G cell line. Fig. 2B shows a similar reduction in SSeCKS/Gravin RNA levels in various human prostate cancer cell lines compared to normal human prostate. Growth of

the androgen-responsive LNCaP cells (32) in the absence of androgens for >9 months had no effect on SSeCKS/Gravin transcript levels, possibly because LNCaP contain a 6q24-ter deletion (33), a region encompassing *gravin* (34; this study). Thus, the loss of SSeCKS/Gravin message is typical in prostate cancer lines.

We then compared the relative levels of SSeCKS/Gravin protein in various untransformed and cancerous prostate cell lines. The P69 series (23) consists of human prostate epithelial lines with increasing oncogenic characteristics in nude mice: P69 are non-tumorigenic cells immortalized with SV40 Tag; M2182 are non-metastatic variants that form tumors at the primary injection site; M12 are variants that form lung metastases following intraperitoneal or intraprostatic injection. Fig. 2C shows a severe decrease (>10-fold) in the levels of the 305/287kD major Gravin isoforms in both M2182 and M12 cells compared to the parental P69 cells suggesting that downregulation occurs prior to the onset of metastatic growth.

Our previous data indicated that the level of SSeCKS RNA and protein increases dramatically under contact-inhibited growth conditions (8,11,30,35). Fig. 2C shows an increase in SSeCKS/Gravin protein levels in increasingly confluent cultures of EP12 cells. In contrast, SSeCKS levels in MLL cells, which are 4-fold lower than EP12 cells, are not affected by culture density. However, the abundance of the 240kD SSeCKS isoform as well as a novel ~80kD isoform increases in MLL following growth at high density (Fig. 2D). The 240kD isoform (250kD in human cells) is a proteolytic cleavage product lacking N-terminal sequences (31). Thus, SSeCKS/Gravin protein levels are induced in both epithelial cells and fibroblasts in response to increased culture density, and this induction mechanism is

inactive in prostate cancer cells. Also, generation of SSeCKS proteolytic fragments increases in MLL cells in response to culture density.

Tet-regulated SSeCKS re-expression suppresses MLL-induced oncogenesis. We demonstrated previously that the tet-regulated re-expression of SSeCKS led to the suppression of *src*-induced oncogenic growth fibroblasts (9). To determine whether SSeCKS suppress MLL-induced oncogenesis, we produced MLL lines with tet-regulated SSeCKS expression. Fig. 3A shows similar levels of SSeCKS protein in the MLL/tTAK cells compared to the parental MLL cells. The MLL/tTAK cells express a tet-regulated form of the tTA transactivator (24) which we found to be much less toxic than the constitutively-expressed tTA. Expression of the 290kD SSeCKS isoform was induced 4-20 fold in several independently derived clones grown without tet (Fig. 3A). These clones also express background levels of SSeCKS in the presence of tet. The ~80kD isoform was present in the MLL/tTAK cells and in all the MLL[tet/SSeCKS] clones grown with tet, whereas tet removal correlated with a 2-10 fold decrease in the abundance of this isoform.

We investigated whether the ~80kD isoform was possibly produced by a factor either present or absent in prostate cancer versus untransformed cells. NP-40 lysates from MLL and EP12 cells were mixed at a 1:1 ratio to determine if factors from one lysate would decrease the presence of the ~80kD isoform. Table 1 shows that mixing of the lysates at 30°C for 5 min caused a decrease in the abundance of the ~80kD isoform (compared to incubation of the MLL lysate alone). Pre-boiling of the MLL lysate did not effect the EP12-induced decrease, whereas boiling of the EP12 lysate did. This indicates that EP12 encodes a heat-labile factor that is antagonistic to the production or stability of the ~80kD isoform.

The MLL[tet/SSeCKS] clones exhibited increased cell flattening, decreased refractility, and increased cell-cell interaction following the removal of tet (Fig.3B). SSeCKS induced a fibroblast-like morphology rather than the epithelial morphology typified by EP12 cells (compare Fig. 4B, panel g and 4C, panel b to 4B, panel i). tTAK caused mild cell flattening (compare Fig. 4B, panel d to 4B, panel e), and although this was less than that induced by SSeCKS (examples: Fig. 4B, panel g and 4C, panel b), it is impossible to exclude that SSeCKS and tTAK work cooperatively.

We analyzed the compartmentalization of SSeCKS by IFA using immunoaffinity-purified anti-SSeCKS Ig (30). Fig. 4A shows enrichment of SSeCKS in the perinuclear regions of EP12 cells as well as a cortical cytoskeletal distribution. SSeCKS was enriched in actin-dense membrane ruffles (Fig. 4A, panels a/a') and in focal complexes connected to actin stress fibers (Fig. 4A, panels b/b'). SSeCKS induced filopodia-like projections (Fig. 4B, panels a-c) with enrichments of SSeCKS in membrane ruffles (Fig. 4B, panels a-c; Fig. 4B, panel g) and at the termini of filopodia (Fig. 4C, panel b). In cells fixed with aldehydes to help retain cytoskeletal filamentation (Fig. 4C, panel c), the nature of the SSeCKS-associated cytoskeletal infrastructure can be appreciated. These effects are strikingly similar to those induced in NIH3T3 fibroblasts following SSeCKS expression (9,26), suggesting conserved functions for SSeCKS in the control of cytoskeletal architecture.

We then addressed whether SSeCKS expression affected parameters of *in vitro* oncogenic growth. Fig. 5A shows that SSeCKS expression decreased proliferation rates and saturation densities 20-70% compared to tTAK control cells. Expression of the tTAK alone (V-2 or V-7) was somewhat inhibitory, probably due to squelching of transcription factors by the VP16 moiety of tTAK (9,26,36). These data indicate that SSeCKS induces

a slight but significant decrease in proliferation rate and saturation density, although, again, these effects cannot be separated from those induced by the tTAK alone.

Fig. 5B shows that SSeCKS expression inhibited anchorage-independent growth 4-5 fold over that induced by tTAK alone. This may be explained by SSeCKS-induced decreases in proliferation (Fig. 5A), and partially by tTAK effects. Thus, we sought to determine whether SSeCKS could inhibit the anchorage-independent growth of MLL in the absence of the tet system. Fig. 5C shows that transient expression of SSeCKS decreased the colony forming efficiency of MLL roughly 3-fold. Thus, the suppressive effects of SSeCKS are separable from those of the tTAK.

Suppression of *in vivo* MLL metastasis by SSeCKS. We addressed whether SSeCKS expression could inhibit either growth of primary tumors or generation of secondary lung metastases. Nude mice were injected in their flanks with 10^5 cells and then maintained on tet in their drinking water until tumors were palpable (2-4 mm), whereupon the tet-water was removed. SSeCKS expression mildly inhibited tumor growth at the primary site in comparison to controls (V-2), most significantly at 8-10 days after initial tumor palpation (Fig. 6A). However, analysis of progressing primary-site MLL[tet/SSeCKS] tumors revealed a loss of tet-regulated SSeCKS expression (Fig. 6C), suggesting that the progressors were variants that had defeated the inducible expression of SSeCKS. Growth of cells *in vitro* from these primary tumors (in DME + hygromycin/puromycin) showed a lack of inducible SSeCKS in the absence of tet (data not shown).

Significantly, the mice receiving the MLL[tet/SSeCKS] cells contained far fewer lung metastases three weeks after primary tumor cell injection than the vector controls (Table 2, Fig. 6B). The inhibition occurred with two independent clones (2-6 and 7-2). Because

expression of SSeCKS had no effect on cell motility in a monolayer wounding assay (Fig. 6D), it is unlikely that these cells are defective for cell motility *in vivo*. These data indicate a role for SSeCKS in tumor suppression, especially at the level of metastasis formation.

Loss of SSeCKS/Gravin expression in well-differentiated human prostate cancer. We analyzed various human prostate lesions for SSeCKS/Gravin expression. SSeCKS/Gravin stained extensively in prostatic epithelial cells, especially the basal epithelial cells, although cell surface staining was detected in some columnar epithelial cells (Fig. 7A, panel a). Abundant SSeCKS/Gravin staining was detected in benign prostatic hyperplastic lesions (Fig. 7A, panel b) and in well-differentiated carcinomas (Fig. 7A, panel c). In contrast, SSeCKS/Gravin staining was absent in undifferentiated carcinomas (Fig. 7A, panel d). Non-cancerous ducts in the same prostate contain epithelia with robust SSeCKS/Gravin staining. A larger survey of human samples showed a consistent loss of SSeCKS/Gravin expression in MPA (Gleason scores >6; Table 3). MPAs were all positive for prostatic acid phosphatase as a control for stainability. These data correlate the loss of SSeCKS/Gravin with the onset of aggressive prostate oncogenesis in humans.

DISCUSSION

We present evidence that the loss of expression of SSeCKS/Gravin correlates with increased oncogenic behavior of prostate cancer in both rodents and humans, and that SSeCKS re-expression suppresses anchorage-independent and metastatic growth. Preliminary evidence indicates a selective loss of SSeCKS/Gravin expression in advanced, undifferentiated human prostate cancers. These data strongly suggest that the loss of SSeCKS/Gravin contributes to the progression of prostate cancer and that SSeCKS/Gravin encode tumor suppressor activity.

Although SSeCKS/Gravin expression is severely decreased in the tumorigenic variants of P69, these cells show no loss of chromosome 6q (23), indicating that the mechanism is most likely transcriptional downregulation. However, unlike human prostate cancer which typically metastasizes to the bone, the metastatic M12 cells were selected for metastasis to the lung. This underlines the dearth of nude mouse/prostate cancer cell models that specifically induce boney metastases.

The ectopic expression of SSeCKS in MLL cells leads to cell flattening, the production of filopodia- and lamellipodia-like projections, and decreases in cell proliferation, saturation densities and anchorage-independent growth. SSeCKS induces similar effects in *src*-transformed NIH3T3 fibroblasts (9). For reasons that are unclear, tTAK can induce partial effects in the MLL system but has no effect in NIH3T3. Thus, some of the SSeCKS-induced effects in MLL may be based on synergy with tTAK. We do show, however, that SSeCKS can suppress anchorage-independent growth in the absence of tTAK

SSeCKS expression antagonizes MLL-induced oncogenesis *in vivo*, especially the generation of lung metastases. The ease at which the MLL cells override the tet-regulated

SSeCKS expression *in vivo* probably belies the plasticity of these tumor cells. Nonetheless, our results suggest that either the sustained expression of SSeCKS or its repeated dosage in prostate tumors would decrease primary tumor growth and metastatic potential.

The association of SSeCKS with cortical, actin-based cytoskeletal structures (26) leads us to believe that the tumor suppressive activity of SSeCKS is a direct results of its ability to reorganize cytoskeletal architecture. Tumor cells often have normal steady-state levels of major cytoskeletal proteins such as actin and tubulin yet have increased turnover of their polymerized, filamentous forms compared to untransformed cells (37). In contrast, cancer cells have decreased levels of minor or intermediate cytoskeletal proteins such as vinculin, α-actinin, tropomyosin or cytokeratins (38,39). Many groups have shown that re-expression of any one of these latter proteins is sufficient to induce a cytoskeletal reorganization that stabilizes microtubule and microfilament structures. SSeCKS seems to function in a similar manner.

We also noted that SSeCKS expression induced increased cell-cell adhesion in most of the MLL[tet/SSeCKS] clones. E-cadherin expression is lost in MLL (40,41), possibly due to deletion of both alleles (42). We could not detect E-cadherin or α-catenin in MLL[tet/SSeCKS] cells even after ectopic SSeCKS expression; β-catenin, which is detectable in parental MLL cells, was not affected by SSeCKS (data not shown). A pan-cadherin polyclonal antibody (Sigma) to E-, EP-, N-, P, L- and V-cadherins failed to show any increase in cadherin levels after SSeCKS expression. Thus, cell-cell adhesion may be facilitated by atypical cadherins or by non-cadherin moieties such as selectins or integrins. The latter notion is strengthened by data that MLL cells show increased levels of β_1 and

α_5 integrins as well as fibronectin (41) which might antagonize mechanisms of normal cell-cell adherence.

An ~80kD SSeCKS isoform was detected in human and rat prostate cancer cells but not their untransformed control lines or in SSeCKS re-expressors. We postulate that untransformed prostate epithelia express a heat-labile factor which either degrades the ~80kD isoform or inhibits a tumor-specific protease that generates the ~80kD isoform. This protease is unlikely a caspase because SSeCKS lacks any of the three recognition motifs (43).

In sum, we have demonstrated that the loss of SSeCKS/Gravin expression is typical in prostate cancer cell lines and in human MPA. We also show that SSeCKS/Gravin can suppress prostate oncogenesis *in vitro* and *in vivo*, suggesting that re-expression would antagonize progression of prostate cancer in patients.

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TABLE 1

Effect of lysate mixing on the generation of the 80kD SSeCKS isoform

Reactants[a]		Presence of 80 kD band	Length of Incubation
MLL lysate	+ EP12 lysate	-	5 min
MLL lysate (boiled)	+ EP12 lysate	-	5 min
MLL lysate	+ EP12 lysate (boiled)	+	>30 min

[a]-- MLL and EP12 lysates were added at a 1:1 ratio.

TABLE 2

Effect of Ectopic SSeCKS Expression on Lung Metastasis Formation

Clone Injected[a]	# of lung metastases[b]	Size of metastases[c]
V-2	32, 6, 3	4 mm ± 1
2-6-11[d]	0, 1, 0	1 mm ± 0.5
7-8-14[d]	0, 0, 0	0
2-6-5[d]	2, 0, 0	1 mm ± 0.5

[a]-- 10^5 cells (>90% viability) injected *s.c.* into flanks.

[b]-- Lung metastases were analyzed 3 weeks after initial appearance of primary tumors.

[c]-- Metastases on the surface of lungs were identified by exclusion of India ink staining as described in Materials and Methods.

[d]-- Subclones of MLL[tet/SSeCKS/ lines 2-6 and 7-8 isolated by single cell cloning methods were identified which had >95% of cells overexpressing SSeCKS (-tet) using IFA analysis (data not shown).

TABLE 3

Loss of Gravin Expression in Malignant Prostate Cancer

Tissue	Gleason score	SSeCKS/Gravin	PAP[a]
normal prostate -1	/	+++	+
normal prostate +2	/	+++	+
BPH -1	/	+++	+
BPH -2	/	++	+
BPH -3	/	+++	+
BPH -4	/	+++	+
BPH -5	/	+++	+
BPH -6	/	++	+
BPH -7	/	+++	+
BPH -8	/	++	+
BPH -9	/	+++	+
BPH -10	/	+++	+
CaP -1	5	+/ -[b]	+
CaP -2	6	+/ -	+
CaP -3	6	+/ -	+
CaP -4	7	-	+
CaP -5	9	-	+
CaP -6	9	-	+
CaP -7	9	-	+
CaP -8	9	-	+
CaP-9	10	-	+

[a] – prostatic acid phosphatase

[b] – staining in neoplastic regions; normal ducts in the same samples displayed typical epithelial cell staining.

FIGURE LEGENDS

Figure 1. Mapping of Gravin to chromosome 6q24-25.2. FISH analysis was performed on metaphase chromosomes of human peripheral blood lymphocytes using a FITC-labeled Gravin cDNA as described in Materials and Methods. The chromosomes were counterstained with DAPI, and the FITC and DAPI images were overlapped to identify specific chromosomes. Note the lone Gravin signal (allele pair) at 6q. Of 100 chromosome sets analyzed, 81% showed hybridization signals, and 10/10 randomly chosen sets showed FITC signals corresponding to region 6q24-25.2 (right panel).

Figure 2. Loss of SSeCKS/Gravin expression in prostate cancer. (**A**) Northern blot analysis of total RNA (25 µg/lane) from Dunning rat prostate cancer cell lines grown in culture or in Copenhagen rats, probed with [^{32}P]-rat SSeCKS cDNA. 28S rRNA, stained with ethidium bromide, is the loading control. (**B**) Northern blot analysis of poly A$^+$ RNA (2 µg/lane) from human prostate cancer cell lines or total normal human prostate, probed with [^{32}P]-rat SSeCKS cDNA. "LNCaP - androgen": two LNCaP cells lines grown without androgens for >9 months. The blot was stripped and reprobed for β-actin as a loading control (below). (**C**) Western blot of protein from various rodent or human prostate cell lines, probed with polyclonal anti-SSeCKS Ig. Confluency: L = low (<40%), M = medium (70-80%), H = high (saturation density). SSeCKS isoforms are identified by arrows relative to protein mol wt markers (290/280kD = doublet arrow; 240kD = triangle). After stripping, the blot was reprobed with anti-vinculin as a loading control. (**D**) High culture density of MLL does not alter the relative abundance of the 290/280kD SSeCKS isoforms but increases the abundance of the ~80kD isoform. Lysates were probed with

anti-actin as a loading control.

Figure 3. Production of MLL cell lines with tet-regulated SSeCKS expression. (**A**) 50 µg of protein from MLL/tTAK, EP12, MLL, or MLL/SSeCKS clones 2-6, 2-7, and 7-2 were immunoblotted for SSeCKS. The 290 and ~80kD SSeCKS isoforms are identified by arrows (right) relative to protein markers (left). Lysates were probed with anti-actin as a loading control. (**B**) Re-expression of SSeCKS suppresses morphological transformation and increases cell-cell adhesion. Phase contrast microscopy of MLL[tet/vector] (V-2) or MLL[tet/SSeCKS] clones (2-4, 2-6, and 2-7) grown in the presence or absence of tet for 3 d. Note the decreased refractility and increased cell flattening in the MLL[tet/SSeCKS] clones grown without tet. All magnifications are 100X.

Figure 4. IFA analysis of SSeCKS and F-actin. (**A**) EP12 cultures fixed in acetone/formaldehyde were stained for SSeCKS (a,b) or F-actin (a'/b') (TRITC-phalloidin). Note the cortical cytoskeletal SSeCKS staining marked by enrichments in the perinuclear region, in membrane ruffles (panels a,b; arrows) and in focal contacts (triangle). (**B**) Ectopic SSeCKS expression induces cell flattening and production of lamellipodia- and filopodia- like projections. Compared to parental MLL grown without tet (d) or MLL[tet/vector] cells grown with tet (f), MLL[tet/vector] cells grown without tet show mild cell flattening (e,h). Growth of MLL[tet/SSeCKS] clones without tet (a, b, c, g) caused more severe cell flattening and production of filopodia-like projections. SSeCKS is enriched at the leading edge of lamellipodia (c; arrows), in membrane ruffles (a, b, g; arrows) and in focal adhesion complexes (a, g; triangles). EP12 (i),

showing typical cell-cell adhesion and epithelial morphology, is shown for comparison. Note the decreased level of SSeCKS staining in the parental or vector-control cells (d, e, f, h) compared to the MLL[tet/SSeCKS] clones. (**C**) MLL[tet/SSeCKS] clone 7-2 grown without tet (b), showing exaggerated projections with enrichments of SSeCKS at their termini (arrows). Growth of these cells with tet results in the typical MLL transformed morphology (a). MLL[tet/SSeCKS] clone 7-2 grown without tet and then fixed in aldehydes/Triton (Materials and Methods) reveals the cortical, SSeCKS-associated cytoskeleton. Size bar for all panels = 20 μm.

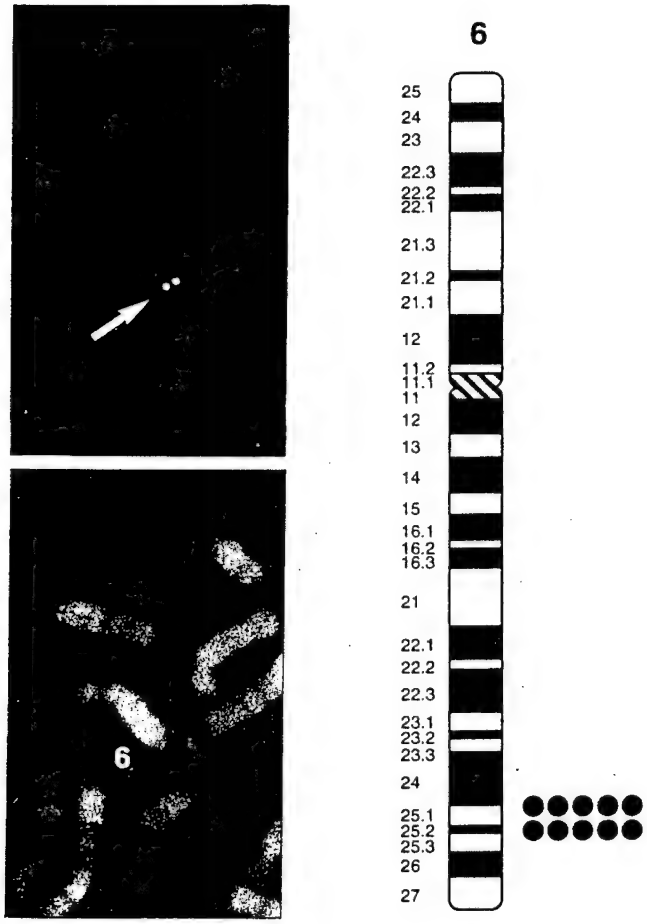
Figure 5. Ectopic SSeCKS decreases MLL proliferation rate and anchorage-independence. (**A**) MLL[tet/vector] control (V-2, V-7) or MLL[tet/SSeCKS] clones were grown in media containing 10% fCS with (solid lines) or without tet (broken lines) and then monitored for proliferation. All experiments were performed in triplicate, and variations at any time point for a given sample were less than 15%. (**B**) Triplicate aliquots of 10^4 MLL[tet/vector] or MLL[tet/SSeCKS] cells were grown in soft agar overlays for 3 weeks at which point the number of colonies formed was determined. In the absence of tet, control cells formed ~40% fewer colonies than if grown with tet. In contrast, the MLL[tet/SSeCKS] clones produced 8-12 fold fewer colonies in the absence of tet. Several SSeCKS-expressor soft-agar colonies were isolated and grown in culture, and all showed a loss of tet-inducible SSeCKS expression (data not shown). (**C**) SSeCKS-induced suppression of anchorage-independence in the absence of tTAK. Triplicate aliquots of MLL cells were transiently transfected with a 1:4 ratio of pBABE*hygro* and either pcDNA3 or pcDNA/SSeCKS, and then grown for three weeks

in soft agar supplemented with hygromycin. Transfection efficiencies were normalized by co-transfecting 1.5 μg of pEGFP and monitoring cell fluorescence.

Figure 6. Effect of ectopic SSeCKS expression on tumor growth in nude mice. (**A**) Athymic *nu/nu* mice (6/experiment) were injected *s.c.* with 10^5 MLL[tet/vector] or MLL[tet/SSeCKS] cells. All mice were maintained on tet/sucrose-water (changed every 2 days). When primary tumors were palpable, half the mice were switched to plain water (-tet) whereas as the others continued with tet-water (+tet). The average sizes of tumors at primary injection sites are shown and the error bars represent the range of tumor sizes in a given cohort. This experiment was repeated twice with two MLL[tet/SSeCKS] clones. The X axis represents the days following initial tumor palpation. (**B**) Relative tumor burden at the primary injection site (○) compared to lung metastases (●). "SSeCKS": results from MLL[tet/SSeCKS] clones 2-6 and 7-2, repeated twice. (**C**) 18-day tumors from mice in the experiment in Panel A were excised and analyzed by western blotting for SSeCKS. Note the loss of tet-regulated SSeCKS expression in MLL[tet/SSeCKS] clone 2-6 from mice initially fed tet and then fed water lacking tet (+/-), compared to 2-6 cells grown in culture. (**D**) Monolayer wounding assay showing no effect of SSeCKS expression on cell motility (clone 2-6) compared to vector (V-2) controls.

Figure 7. Loss of SSeCKS/Gravin in advanced human prostate cancer *in situ*. Formalin-fixed sections of human prostate representing normal tissue (a), benign prostatic hyperplasia (b), well differentiated prostate cancer (c) and advanced, undifferentiated prostate cancer *in situ* (d) were processed and stained by immunohistochemistry for

SSeCKS/Gravin protein as described (31). Size bars equal 10 μm.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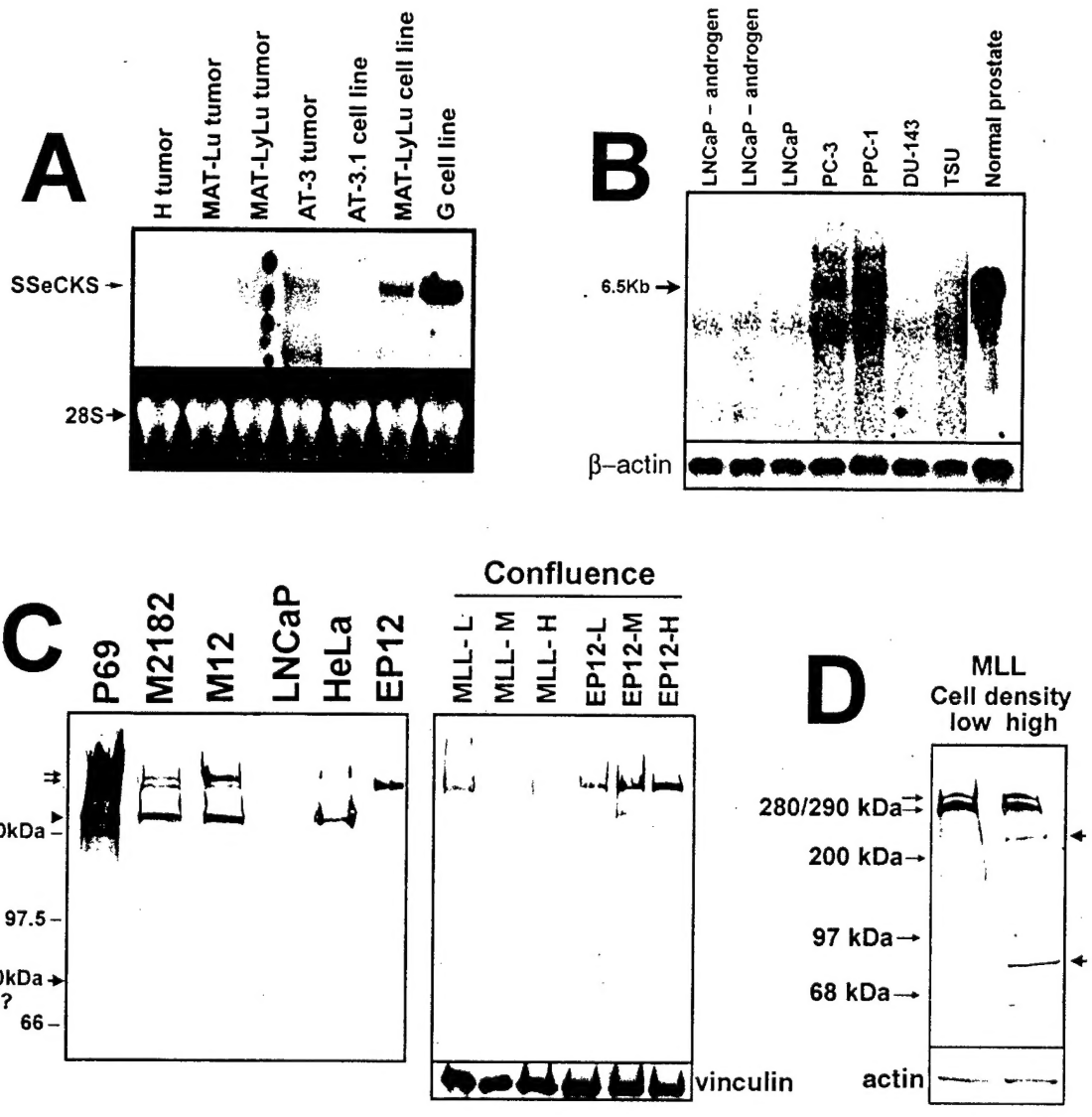
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LNCaP – androgen
LNCaP
PC-3
PPC-1
DU-143
TSU
Normal prostate
6.5Kb
β–actin
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M2182
M12
LNCaP
HeLa
EP12
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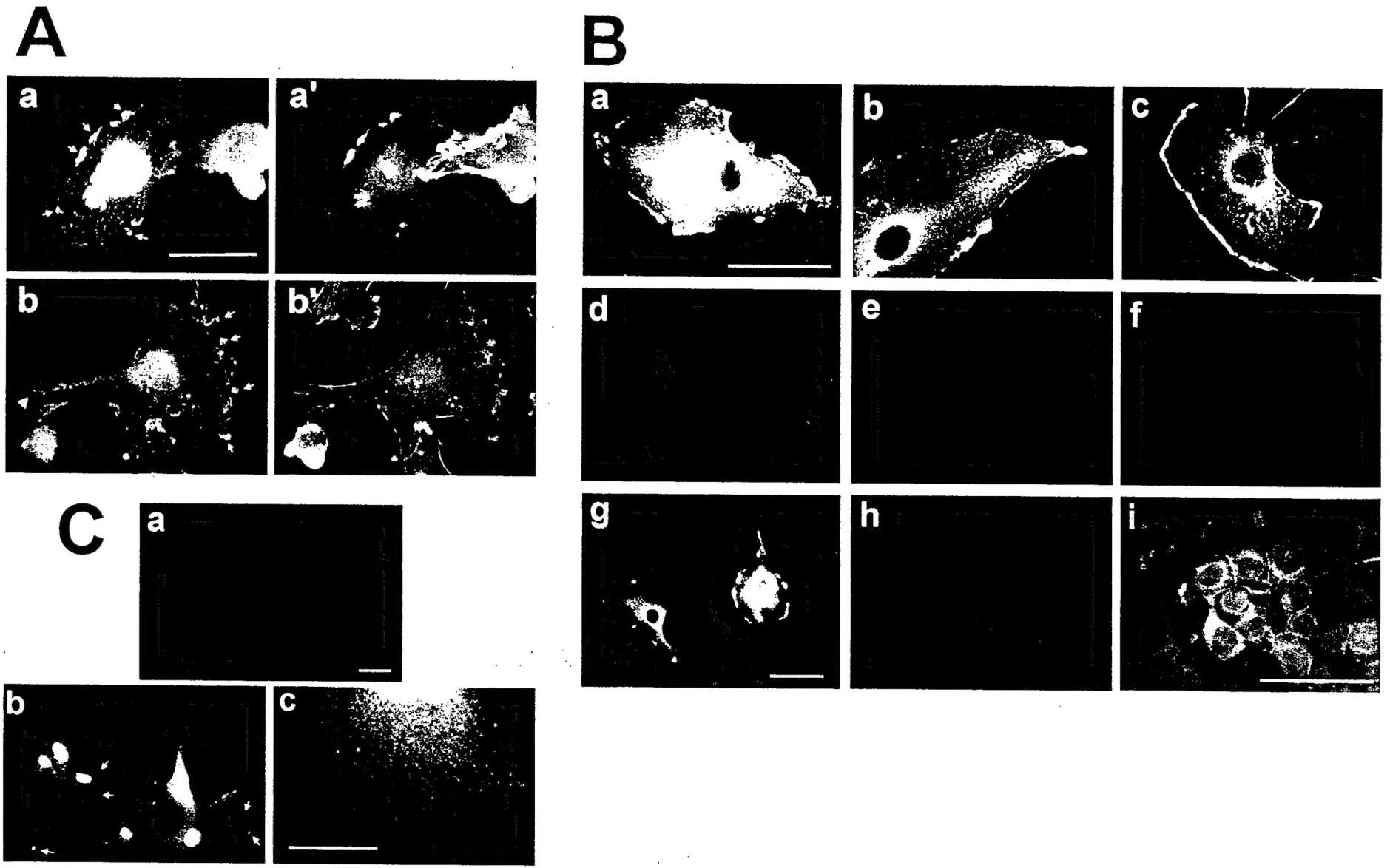
actin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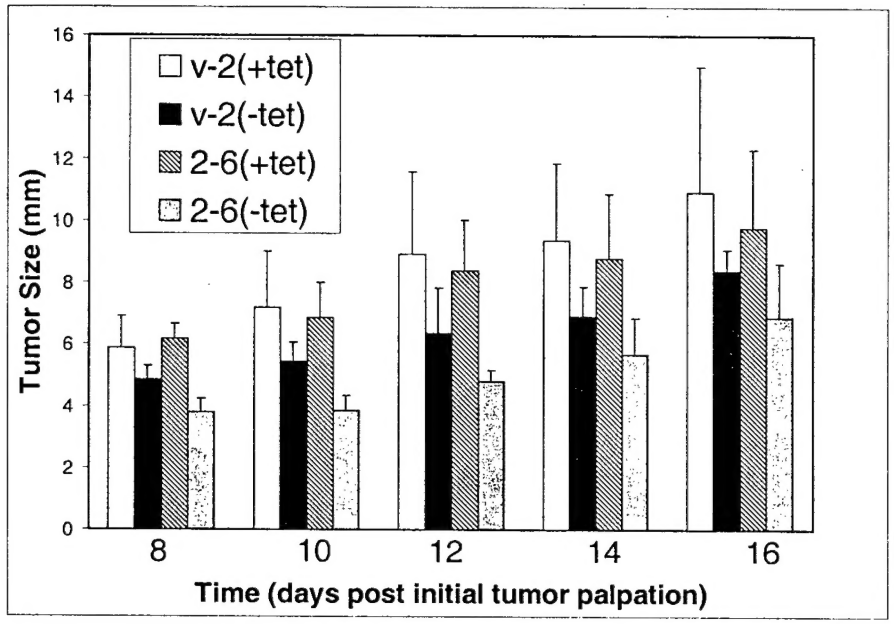
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v-2(+tet)
v-2(-tet)
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2-6(-tet)
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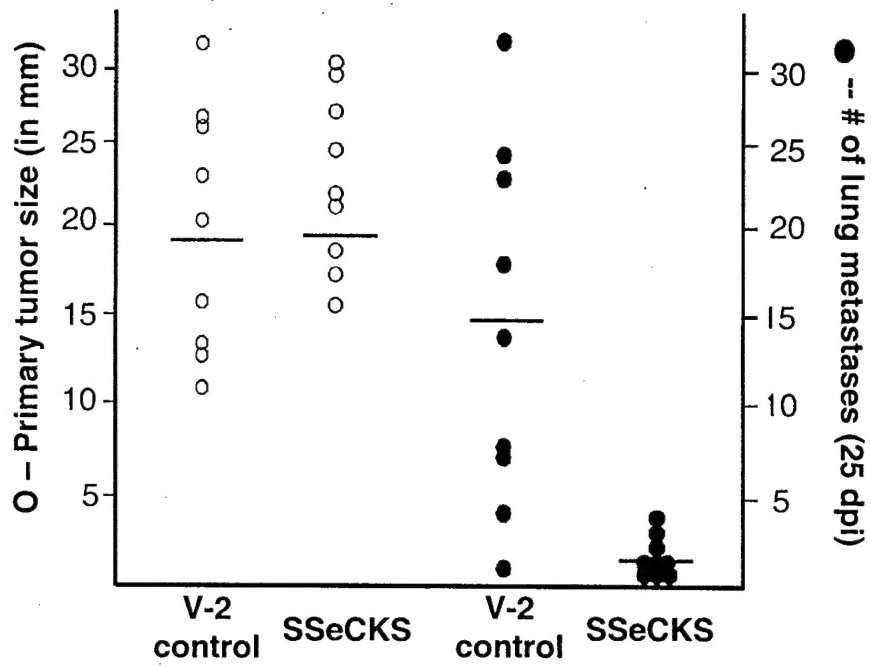
Time (days post initial tumor palpation)


B
O – Primary tumor size (in mm)
● -- # of lung metastases (25 dpi)
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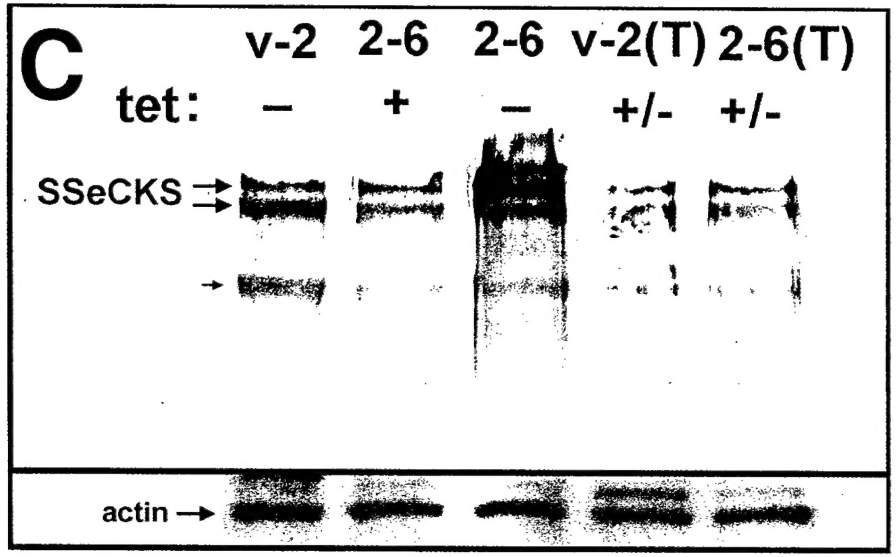
V-2 control
SSeCKS
V-2 control
SSeCKS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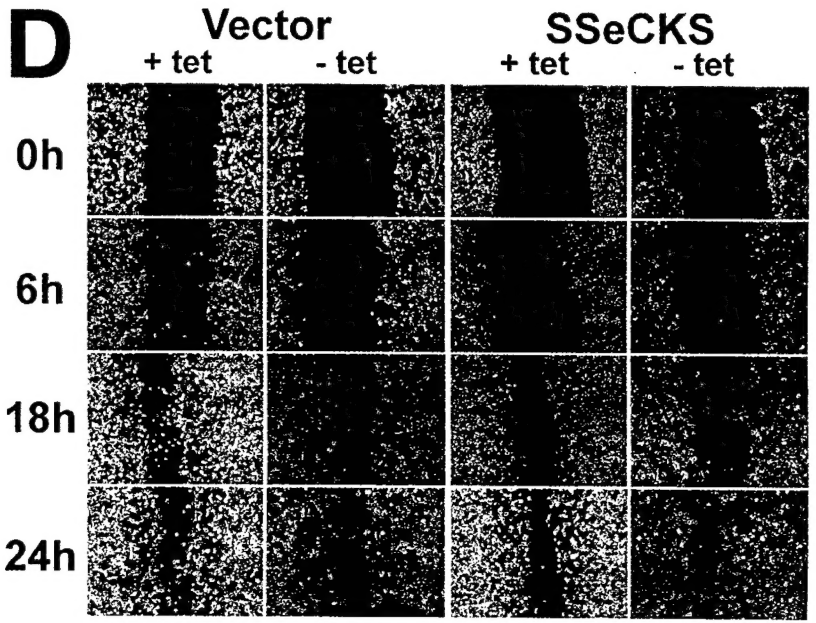
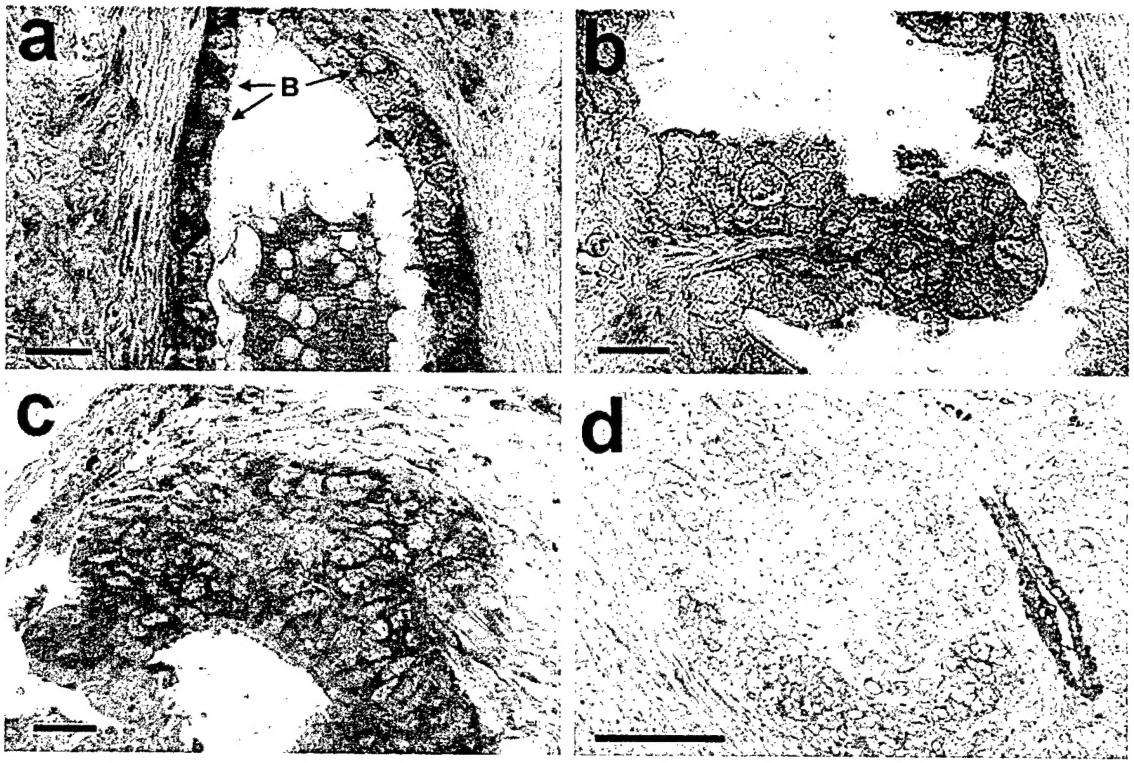
SSeCKS
actin


D
Vector
SSeCKS
+ tet
- tet
+ tet
- tet
0h
6h
18h
24h


a
B
b
c
d